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RESISTANCE OF JUVENILE *ACTINIA TENEBROSA* (CNIDARIA : ANTHOZOA) TO DIGESTIVE ENZYMES

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ABSTRACT

Juvenile *Actinia tenebrosa* are often found brooded in the coelenteric cavities of adults in interseptal spaces. Hence the juvenile sea anemones are spatially separated from the region of food digestion and high enzyme concentrations at the base of the stomodaeum of the adult. Histological examination of juveniles experimentally subjected to digestive enzymes indicated that ectodermal tissue of juveniles is resistant to crude preparations of the proteolytic enzymes trypsin, chymotrypsin and papain, but at the same time endodermal tissue is partly digested by chymotrypsin and extensively digested by papain. Following incubation with hyaluronidase, ectoderm is not digested by trypsin, is partly digested by chymotrypsin, and is extensively digested by papain. Trypsin is probably the main digestive protease in coelenterates and these simple introductory experiments suggest that the structural proteins of *Actinia* are resistant to trypsin digestion. Mucus may protect juveniles from other gastric enzymes and there may be a further protective layer, which is not protein, on the ectodermal surface.

INTRODUCTION

The intertidal sea anemone *Actinia tenebrosa* Farquhar, 1898 broods post-Edwardsia stage juveniles in the coelenteron (Farquhar 1898; Parry 1951; Ottaway and Thomas 1971; Ottaway 1973) and a similar habit has been recorded for many other anemones, including the northern hemisphere *Actinia equina* L. (Dalyell 1848; Gosse 1860; Stephenson 1928, 1935; Chia and Rostron 1970). Enzymatic activity of coelenteric fluid is low for *Metridium marginatum* (Bodansky 1923), *Actinia mesembryanthemum* (Ishida 1936), *Pseudactinia flagellifera* (Krigjsman and Talbot 1953), and *Calliactis parasitica* (Nicol 1959), and this is probably so for *Actinia tenebrosa*. Extracellular digestive enzymes are secreted directly onto food and contained there by mucus, which largely prevents enzymes mixing with coelenteric fluid. Thus, brooded juveniles are normally exposed to only very low concentrations of digestive enzymes; however, brooded juvenile *A. equina* and *A. tenebrosa* have been observed feeding directly on food captured and ingested by the host adult (Chia and Rostron 1970; pers. obs.). Under these circumstances juveniles may be subjected to high concentrations of enzymes, as the adult digests the food.

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This paper examines the spatial relationships between food and juveniles within the coelenteric cavities of adult *A. tenebrosa* and the effects, *in vivo* and *in vitro*, of digestive enzymes on juveniles.

METHODS AND MATERIALS

COLLECTION AND TREATMENT OF ANIMALS

Anemones were collected at low tide from boulders of the Outer Harbour breakwater, South Australia, during the period January 1969 - July 1969, as described by Ottaway and Thomas (1971), and were placed into a recirculated seawater system within several hours of collection. Most anemones were used between 24 and 48 h of collection and none were fed before experiments. Seawater, of 38‰ salinity, was collected and filtered immediately before use.

For histological examination anemones were anaesthetized in 1:1 8% $MgCl_2.6H_2O$: seawater (Batham, Pantin and Robson 1960), fixed in Bouin's solution for about 18 h, washed in 50% ethanol and stored in 70% ethanol. Tissues were embedded in paraffin wax, sectioned at 12 µm, and stained with Alcian Blue 8G/Chlorantine Fast Red 5B (Lison 1954). Whole adults were sectioned at 2-4 mm thickness after embedding in gelatine (Cowdrey 1952).

MATERIALS FOR *IN VITRO* EXPERIMENTS

Trypsin (from hog pancreas; Sigma Chemical Co.: T8128; Type II, crude); Chymotrypsin (from bovine pancreas; Sigma Chemical Co.: C4754; Type II); Papain (from papaya juice; British Drug Houses; biochemical, crude); Hyaluronidase (from bovine testes; Sigma Chemical Co.: H2001; Type I).

EXPERIMENTAL PROCEDURE AND RESULTS

1. SPATIAL RELATIONSHIPS BETWEEN FOOD AND JUVENILES IN THE COELENTERIC CAVITY

Adult *A. tenebrosa* were fed pieces of fish which had been covered with finely ground animal charcoal to identify their position in sectioned anemones. After 4-8 h the anemones were fixed, and some were immediately sectioned whilst others were sectioned after embedding in gelatine. Forty-five adults were found to contain juveniles as well as food; of these, fifteen adults had been embedded in gelatine before sectioning. In all adults food was found in a bolus at the base of the stomodaeum. Juveniles were usually found in peripheral regions of the coelenteron, especially in the interseptal spaces between stomodaeum and column wall, although in two adults juveniles were in contact with the food and apparently had been ingesting it when fixed. Microscopic examination of sections of these juveniles revealed no tissue breakdown.

2. RESISTANCE OF JUVENILES TO DIGESTION BY ADULT *A. TENEBROSA*

Adult *Actinia* were induced to ingest juveniles by offering these surrounded by small pieces of fish flesh. After 8 h, balls of mucus were egested which were found to contain scant fish remains and the unharmed juveniles.

3. RESISTANCE OF JUVENILES TO DIGESTION BY ADULT *ISANEMONIA AUSTRALIS*

Isanemonia australis Carlgren is a subtidal anemone found in the same vicinity as *A. tenebrosa* at Outer Harbour. When one species contacts the other, individuals always react aggressively, and sometimes kill each other. Adult *Isanemonia* readily ingests juvenile *Actinia*.

One large adult *I. australis* was starved for 3 days, then fed in succession a few small pieces of fish, 3 juvenile *Actinia* (3 mm oral disc diameter), and a crab's maxilliped weighing about 1 g. After 6 h the juvenile *Actinia* were egested dead and highly contracted. After a further hour, remains of the fish and maxilliped were egested, both with very little flesh remaining. The juveniles were fixed, embedded and sectioned. Microscopic examination of sections revealed only slight breakdown of tissues. (Fig. 1a).

4. RESISTANCE OF JUVENILES TO DIGESTION BY ENZYMES *IN VITRO*

Characterization of coelenterate digestive enzymes suggests that extracellular proteases are present which are similar to mammalian trypsins and chymotrypsins. Krijgsman and Talbot (1953) isolated trypsin-like proteases from *Pseudactinia flagellifera*; Gibson and Dixon (1966, 1969) found three proteases in *Metridium senile* with properties very similar to those of α-chymotrypsins from bovine pancreas. Coan and Travis (1970) examined proteases from the sea pansy (*Renilla reniformis*: Pennatulacea) and found a trypsin analogous in structure and function to trypsins from other animals, and also a chymotrypsin-like protease.

Preliminary testing of the coelenteric fluid from fed and unfed *A. tenebrosa* showed that pH is usually 7.4-7.6. This is within the ranges found by Krijgsman and Talbot (1953) for *Pseudactinia flagellifera* (pH 6.7-7.8) and Vader and Lonning (1973) for *Bolocera tuediae* (pH 7.3-7.6). Juveniles were incubated in enzyme solutions at pH 7.2-7.8, according to the enzyme in use for 3 h at 20°C. Enzyme and buffer solutions were made up immediately before experiments in filtered seawater, unless otherwise stated.

i. Effect of hyaluronidase

Treatments are shown in Table 1. There were 5 replicates of each treatment, with one juvenile in each replicate. Hyaluronidase was denatured by boiling at 100°C for 15 minutes. Juveniles were incubated for 14 h at 20°C, and each solution was thoroughly agitated and aerated about every three hours.

All juveniles were alive after 14 h and had attached to the insides of the incubation tubes. Two animals from each treatment were sectioned and examined. The anemones treated with active hyaluronidase showed no surface mucus but were otherwise normal. Anemones from control treatments had much loose, patchy, surface mucus. Thus, hyaluronidase is probably effective in removing at least some surface mucus from juveniles but it does not disrupt their tissues or dissociate cells.

TABLE 1. INCUBATION OF JUVENILE *ACTINIA* WITH HYALURONIDASE

	1% Hyaluronidase in seawater (w/v)	0.2 M-Tris-HCl (pH 7.4) in distilled water	Filtered seawater
Test	2 ml active	0.5 ml	1.5 ml
Control 1	2 ml denatured	0.5 ml	1.5 ml
Control 2	-	0.5 ml	3.5 ml

ii. Effect of trypsin

Preliminary testing of the coelenteric fluid from adult, fed *Actinia*, indicated that proteolytic activity was equivalent to that of a 0.05% solution of trypsin. Treatments for this experiment are shown in Table 2: 2.5 ml trypsin solution and 0.5 ml 0.1M Tris-HCl pH 7.8 were added to tubes containing either 3 healthy juveniles or 50 mg ± 1 mg fibrin stained with congo red (Krijgsman and Talbot 1953). Digestion of the fibrin substrate releases congo red into solution, with intensity of dye being proportional to proteolytic activity. Juveniles and fibrin were incubated for 3 h at 20°C.

TABLE 2. INCUBATION OF JUVENILE *ACTINIA* OR FIBRIN IN TRYPSIN SOLUTIONS FOR 3 H AT 20°C

Trypsin (% w/v)	Total number of juveniles incubated	Appearance of juveniles after incubation	Standards* (amount of dye released from fibrin)
1	6	contracted, but alive	.80, .60
0.5	3	slightly contracted, alive	.59
0.1	3	expanded, alive	.47
0.05	3	expanded, alive	.40
seawater and Tris buffer only	3	expanded, alive	.00

* Figures are absorbance of solution in a Beckman-Spinco 151 Spectrocolorimeter set at 670 nm.

After 3 h incubation, pH of the 1% and 5% trypsin solutions was 6.0, but pH of the other solutions remained unaltered. All juveniles seemed healthy, and those from 1% and 0.5% solutions were sectioned.

iii. Effect of hyaluronidase followed by trypsin

Juvenile *Actinia* were incubated for 3 h at 20°C in the solutions shown below (Table 3). Juveniles incubated in hyaluronidase were then incubated for a further 3 h at 20°C in trypsin solution: 3 animals in 1% trypsin, 3 animals in 0.5% trypsin, as described above (Expt 4.ii). Juveniles incubated initially in buffered seawater were subsequently incubated for a further 3 h at 20°C in Tris-HCl in seawater (pH 7.8).

TABLE 3. INITIAL TREATMENT OF JUVENILES WITH HYALURONIDASE

	1% hyaluronidase in seawater	0.2 M Tris-HCl in distilled water (pH 7.4)	Seawater
Test treatments:			
6 replicates - one juvenile in each	2 ml	1 ml	1 ml
Control treatments:			
3 replicates - one juvenile in each	-	1 ml	3 ml

All animals were sectioned and examined. There was no obvious digestion of tissues, nor was there any marked difference between juveniles incubated in the enzyme solutions and those incubated in buffers only (Figs 1b, 1c).

iv. Effect of chymotrypsin

Preliminary testing of the coelenteric fluid from adult fed *Actinia* indicated that proteolytic activity was equivalent to 0.005% chymotrypsin solution. Immediately before use 0.1% chymotrypsin solution was made up in 0.5M Tris-HCl in seawater (pH 7.7). This was serially diluted to give the concentrations shown below (Table 4). Juveniles were placed into 2 ml of each solution, and a further 2 ml of each solution was placed onto 50 mgm of fibrin/congo red. Treatments were incubated for 3 h at 20°C.

TABLE 4. INCUBATION OF JUVENILE *ACTINIA* OR FIBRIN/CONGO RED IN CHYMOTRYPSIN SOLUTIONS FOR 3 H AT 20°C

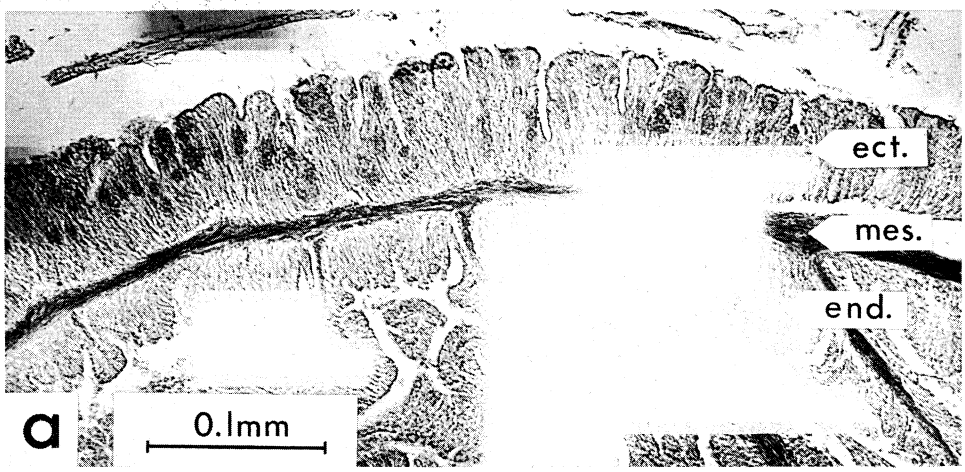
Chymotrypsin (% w/v)	Total number of juveniles incubated	Appearance of juveniles after incubation	Standards* (amount of dye released from fibrin)
0.1	3	expanded	.67
0.05	3	expanded	.57
0.01	3	expanded	.35
0.005	3	expanded	.15
buffer solution only	3	expanded	.15

* Absorbance at 670 nm.

Examination of sections showed that the ectodermal tissue was largely intact, whilst the endodermal tissue had been partly digested with considerable loss of cellular detail.

v. Effect of hyaluronidase followed by chymotrypsin

Juveniles were treated with hyaluronidase (as in Expt 4.iii), and then treated with chymotrypsin (as in Expt 4.iv). There was no obvious difference between sections of these juveniles and those sections of juveniles only treated with chymotrypsin: endodermal tissue was partly digested while ectodermal tissue was apparently intact (Fig. 2a).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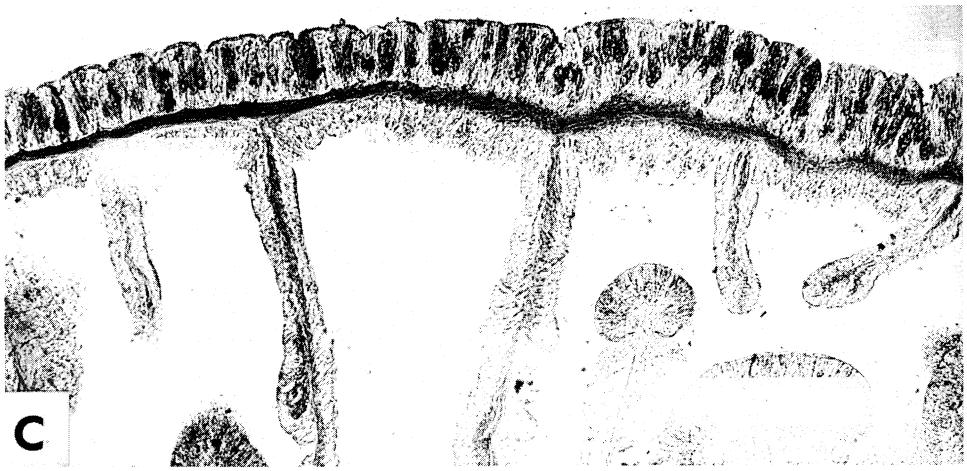
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vi. Effect of Papain

Fifteen minutes before use, 12 ml 2% papain solution was activated with 5 ml 0.05 M cysteine. Juveniles were then incubated for 3 h at 20°C in 2 ml activated papain, 1 ml 0.1 M Tris-HCl in seawater (pH 7.2), and 2 ml seawater. Examination of sectioned juveniles showed extensive breakdown of endoderm, which was completely digested in some regions (Fig. 2b). Ectoderm was virtually intact.

vii. Effect of hyaluronidase followed by papain

Juveniles were treated with 0.25% hyaluronidase in buffered seawater (as in Expt 4.iii), then with papain (as in Expt 4.vi). The effect on endoderm was essentially as the effect of papain alone; however, ectoderm was now also extensively digested. Histological examination of tissues suggested that hyaluronidase removed surface mucus from secreting gland cells, and then papain penetrated gland cells to digest underlying protein and adjacent cells (Fig. 2c).

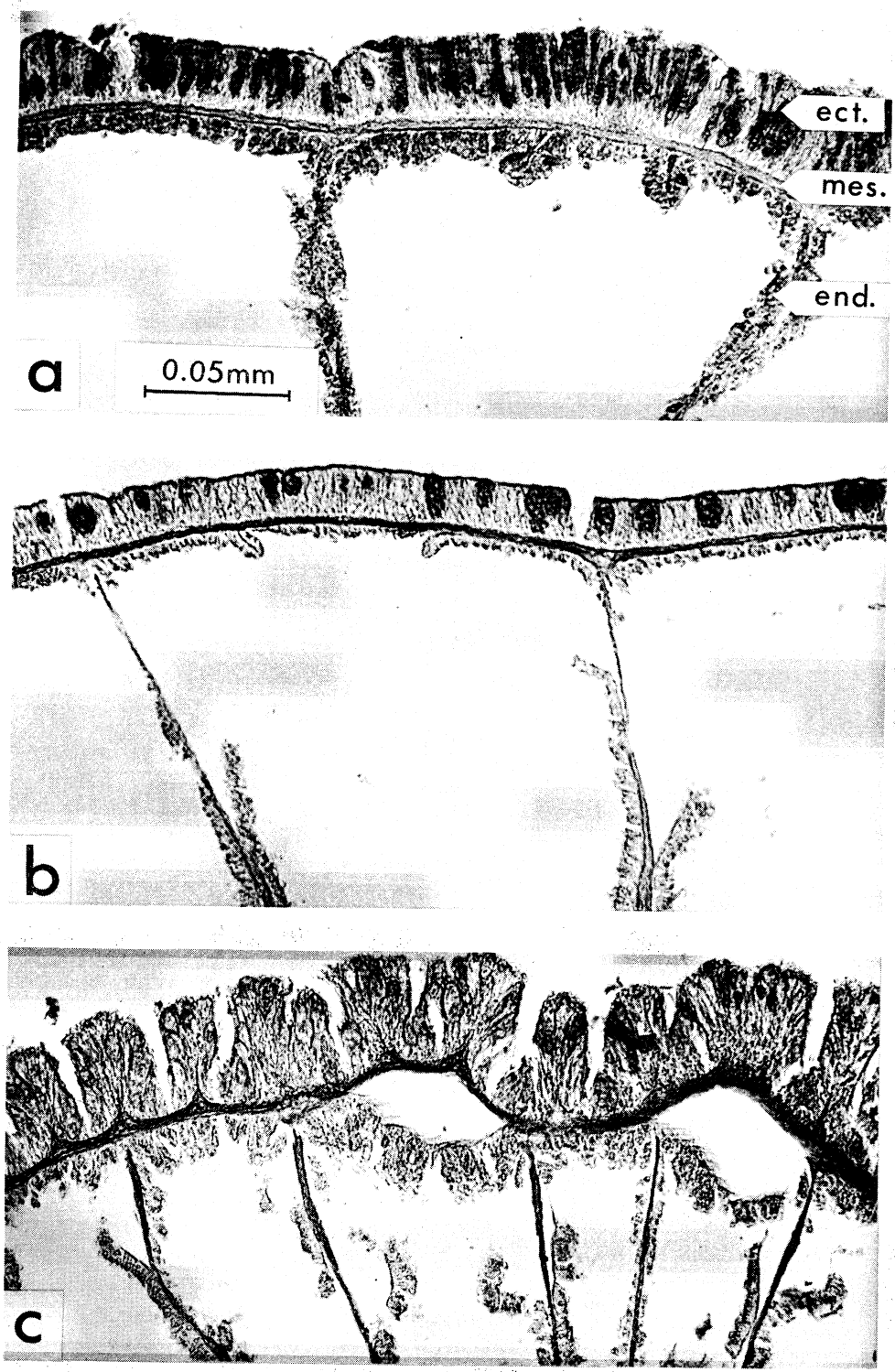
DISCUSSION

Digestive efficiency is high in sea anemones and these animals quickly break down and absorb food, even though the proteolytic activity of coelenteric fluid is low. This apparent contradiction was convincingly explained by Nicol (1959) who found that mesenterial filaments effectively localize extracellular digestive enzymes around food at the base of the stomodaeum. Filaments surround ingested food and secrete enzymes and mucus directly onto it, so that mucus envelops the food and contains the large enzyme molecules, of molecular weight about 24,000. Some breakdown products of food, are absorbed and phagocytosed by mesenterial filaments while some soluble food molecules diffuse through the mucus and dissolve in the coelenteric fluid.

Fig. 1a. Transverse section (12 µm) through a juvenile *Actinia*, showing the effect of feeding the juvenile to an adult *Isanemonia australis*. The juvenile was egested dead and highly contracted, but tissues showed little evidence of breakdown. Mesogloea is stained with Chlorantine Fast Red 5B, mucus and mucous gland cells are stained with Alcian Blue 8G. ect = ectoderm, mes = mesogloea, end = endoderm.

Fig. 1b. Effect of incubating juvenile *Actinia* with buffer solutions used during *in vitro* enzyme experiments. All juveniles were alive after incubation, and tissues appeared intact. Staining and scale as for Fig. 1a.

Fig. 1c. Effect of incubating juvenile *Actinia* with 0.5% hyaluronidase then 0.85% trypsin. All juveniles were alive after incubation, and tissues appeared intact. Staining and scale as for Fig. 1a.


ect.
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end.
a
0.05mm
b
c

Brooded juvenile *Actinia tenebrosa* usually develop in the interseptal spaces away from the immediate region of food digestion in the host adult, and recent work suggests juveniles may absorb food molecules directly from coelenteric fluid (Chia 1972). In some instances, however, brooded juveniles actually ingest food captured by the adult while the food is presumably being digested. Thus, juveniles which are normally subjected to only very low concentrations of digestive enzymes may sometimes be exposed to high concentrations and yet still survive unharmed. Much more work is needed, however, to characterize enzymes in the coelenteric fluid and food bolus and determine relative concentrations.

When live juveniles are surrounded by fish flesh and fed to adult *Actinia*, the food is digested but the juveniles are neither digested nor killed. Juveniles must therefore possess some resistance to the digestive enzymes of adult *Actinia* and immunity against nematocysts and toxins in mesenterial filaments. When live juveniles and food are fed to adult *Isanemonia australis*, food is digested while juveniles are not, but juveniles are egested dead. Adult *A. tenebrosa* and *I. australis* react aggressively and inflict considerable mutual damage when they meet, and it therefore seems likely that juvenile *Actinia* would be killed soon after capture by nematocysts of *Isanemonia*. Hence, juvenile *Actinia* probably resist digestion passively; that is, resistance is a property of tissues retained for at least several hours after death of juveniles.

Gibson and Dixon (1966, 1969) and Coan and Travis (1970) suggested that the main extracellular digestive enzymes in coelenterates are trypsins and chymotrypsins similar in function and activity to those from mammals. In order to examine the *in vitro* effects of proteases on juveniles, these anemones were incubated with readily-available hog trypsin and bovine chymo-

Fig. 2a. Transverse section (12 μm) through a juvenile *Actinia*, showing the effect of incubation with 0.5% hyaluronidase followed by 0.1% chymotrypsin. All juveniles were alive after incubation and responded to physical stimulation. Ectodermal tissue was largely intact, but endodermal tissue showed cellular breakdown. Staining and scale as for Fig. 1a. ect = ectoderm, mes = mesogloea, end = endoderm.

Fig. 2b. Effect of incubating juvenile *Actinia* with 0.3% papain. Juveniles showed no responses to physical stimulation and endoderm was extensively digested. Ectoderm appeared intact. Staining and scale as for Fig. 1a.

Fig. 2c. Effect of incubating juvenile *Actinia* with 0.5% hyaluronidase followed by 0.3% papain. Juveniles appeared to be dead after incubation. Both endoderm and ectoderm were extensively digested, but the ectoderm appeared to have been attacked at specific sites. Staining and scale as for Fig. 1a.

trypsin, on the assumption that these would function in the same manner as *Actinia* trypsin and chymotrypsin. Papaya papain was also used, since papain has very low substrate specificity and produces more extensive degradation of protein substrates than either trypsin or chymotrypsin (Smith and Kimmel 1960). These proteases are all quite active at pH 7.4-7.6 (Desnuelle 1960), which is the pH range of coelenteric fluid.

In vitro incubation of anemones with trypsin, chymotrypsin and papain, used individually, showed negligible effects on ectoderm. Trypsin also had negligible effects on endoderm, but chymotrypsin partly digested endoderm and papain extensively digested endoderm. Only papain produced a different effect on juveniles treated with hyaluronidase: endodermal and ectodermal tissue were extensively digested, but not in the same manner (Fig. 2c). These results suggest that the structural proteins of juvenile dermal layers are resistant to trypsin digestion and also have some resistance to chymotrypsin digestion. Since it is unlikely that ectodermal protein is passively resistant or contains inhibitors specific for the three proteases, it is necessary to postulate the presence of one or more layers that protect juveniles. Removal of mucus allowed papain to attack ectoderm at what appeared to be specific sites. It is suggested therefore that there is a non-protein layer on the ectodermal surface and that papain entered mucous gland cells through sites opened by hyaluronidase to subsequently digest protein adjacent to these cells.

Brooded juveniles that encounter high concentrations of a host adult's digestive enzymes should survive unharmed provided that the coelenteric cavities of such juveniles did not become flooded with concentrated enzymes from the adult. After a maximum period of about 8 h these juveniles would probably be egested with food remains.

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